in vivo *22*: 587-592 (2008)

Erythropoietin and Growth Factors Exhibit Differential Angiogenic Potential in Mouse Heart

OLGA PAGONOPOULOU[1], ANNA EFTHIMIADOU[1], MARIA LAMBROPOULOU[2], NIKOS PAPADOPOULOS[2] and NIKOS K. NIKOLETTOS[1]

Departments of [1]Physiology and [2]Histology – Embryology, Democritus University of Thrace, Medical School, Alexandroupolis, 68100, Greece

Abstract. *Background: The possible angiogenic effect of recombinant human erythropoietin (rHuEpo) and several possibly angiogenic cytokines such as basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF) and vascular endothelial growth factor (VEGF) was investigated in mouse heart. Materials and Methods: Mice were divided into five groups (n=7/group): A, control; B, rHuEpo-treated; C, (aFGF-treated); D, (VEGF-treated); E, (bFGF-treated). The antibody mouse anti-human CD31 was used to evaluate the vessels present in histological preparations. Results: The results show a significant increase of the vessel number per optical field in the rHuEpo-treated, the bFGF-treated and the VEGF-treated animals compared to controls whereas aFGF did not show any significant angiogenic activity. Conclusion: Erythropoietin has a significant angiogenic effect in the mouse heart, similar to the effect of other angiogenic factors such as bFGF and VEGF whereas aFGF does not exhibit any effect.*

Erythropoietin (EPO) is a hematopoietic factor regulating the proliferation and differentiation of erythroid precursor cells (1). The biological effects of EPO are mediated by its specific interaction with its cell surface receptor EPO-R, a type 1 cytokine receptor that is present in erythroid progenitor cells. EPO-R is also expressed by a variety of nonhematopoietic cell types, including endothelial cells (2), neurons (3) and trophoblast cells (4).

Although the specific functions of EPO/EPO-R in these sites are not yet completely clarified, there is increasing evidence suggesting a wider biological role of EPO/EPO-R not related to erythropoiesis. Among the extrahematopoietic functions of EPO, angiogenesis, the process through which new blood vessels arise from preexisting ones, has been indicated (5). The potential role of EPO in angiogenesis may be considered as a subset of its possible function in improving overall tissue oxygenation and its antiapoptotic role (5).

Recombinant human erythropoietin (rHuEPO) induces a proangiogenic phenotype in human endothelial cells, including both early (*i.e.* increase in cell proliferation and matrix metalloproteinase-2 production) and late (differentiation into vascular tubes) angiogenic events. *In vivo*, in the chick embryo CAM assay, the angiogenic activity of rHuEPO was similar to that exerted by basic fibroblast growth factor (bFGF), a well-known angiogenic cytokine and endothelial cells of the CAM expressed EPO-R, which were co-localized with factor VIII positivity (6).

Fibroblast growth factors (FGFs) available today for therapy in clinical conditions associated with ischemia represent a hopeful strategy for restoration of blood flow and require further evaluation before extensive clinical use. FGF is a family of at least 21 peptides exerting angiogenic properties. Initially, FGF was isolated in the brain by Gospodarowicz in 1974 (7). Since then, many peptides of similar structure have been found to manifest angiogenic properties such as acidic FGF (aFGF), a 140 amino acid polypeptide possessing 55% homology with bFGF which consists of 154 amino acid residues. Both peptides are expressed in a variety of tissues including brain, pituitary, myocardium, kidney and in liver, as well as in macrophages, endothelial and muscle cells. FGFs act as protein mitogens, stimulating angiogenesis under various physiological or pathological conditions. Physiologically, they are also implicated in brain development, cartilage formation, soft tissue repair, migration and differentiation of cells of mesenchymal or neuroectodermal origin. These factors are also implicated in pathological processes, such as neoplasms, as they induce the neoangiogenesis required for tumor growth (8, 9).

The vascular endothelial growth factor (VEGF) family includes six known members: VEGF, placenta growth factor (PIGF), VEGF-B, VEGF-C, VEGF-D and VEGF-E (10-11). VEGF mRNA is induced by hypoxia in cultured cells and up-regulated in tumor cells adjacent to necrotic areas, as

Correspondence to: Olga Pagonopoulou, Department of Physiology, Democritus University of Thrace, Medical School, Alexandroupolis, 68100, Greece. Tel: +30 2551030525, Fax +30 2551030538, e-mail: opagonop@med.duth.gr

Key Words: Angiogenesis, erythropoietin, growth factors, mouse heart.

revealed by *in situ* hybridization. As a secreted endothelial cell-specific mitogen, VEGF is also a candidate for ischemia-induced angiogenesis in pathophysiological conditions in tissues (12). Unlike the FGF family, VEGF is a more specific mitogen for endothelial cells and has the advantage of being secreted by intact cells. Therefore, VEGF may be superior to FGF as a therapeutic agent. Gene transfer is also being widely investigated to determine its applications to angiogenesis.

The use of recombinant growth factors such as FGF or VEGF for therapy in clinical conditions associated with ischemia thus represents a hopeful strategy for blood flow restoration.

We recently reported the angiogenic effects of aFGF, bFGF and VEGF in normal adult rat heart (13-14). Based on these findings, we carried out this study to investigate the angiogenic potential of rHuEPO on endothelial cells of normal mouse myocardial tissue. In addition, we compared the angiogenic potential of rHuEPO to that of the other well-known angiogenic cytokines aFGF, bFGF and VEGF.

Materials and Methods

Experimental design. Thirty-five male BALB-c mice weighing 20-23 g were used. They were housed in individual cages in a temperature-and light-controlled environment, and were allowed free-access to water and laboratory chow. Mice were assigned to the following experimental groups: Group A (Control) consisted of 7 mice, which were used as controls to which 0.1 ml saline was administered intramuscularly (IM) under ether anesthesia, every three days, for a total period of 15 days. Group B (rHuEPO) consisted of 7 mice which received 7000U rHuEPO per week by intraperitoneal (IP) injection for 15 days. Group C (bFGF) consisted of 7 mice which received 1 μg angiogenic factor bFGF intramuscularly under ether anesthesia every three days for 15 days. Group D (aFGF) consisted of 7 mice which received 1 μg angiogenic factor aFGF intramuscularly under ether anesthesia according to the same protocol followed for group B. Group E (VEGF) consisted of 7 mice which received 1 μg angiogenic factor VEGF intramuscularly under ether anesthesia every three days for 15 days.

The dosage and the method of bFGF (15), aFGF (16), VEGF (17) and rHuEPO (18) administration were chosen based on previous reports.

On the 16th day after the onset of the experiment, mice were anesthetized with ether, the thorax was opened through a midline incision and hearts were removed and processed for histological examination. The animals were then euthanized by deep anesthesia with ether.

Drugs. bFGF, aFGF and VEGF were purchased from Cytolab/Peprotech Asia. Recombinant erythropoietin was purchased from Ortho Biotech, Germany.

Histological examination. Tissue specimens from the animals were fixed in neutral buffered formalin (10%) and embedded in paraffin according to standard histological procedures. Four-micron sections of paraffin embedded tissue samples from each case were sectioned on microscope slides, deparaffinized and rehydrated. Slides were then stained with conventional hematoxylin and eosin (H&E). Unstained slides were used for the detection of CD31 monoclonal mouse anti-human antibody (CD31, clone: INN-PECAM-1; NOVOCASTRA, UK) which recognizes the surface antigen CD31 of endothelial cells.

Table I. *Comparison of the vessel number per optical field (p.o.f.) in the various experimental groups*.

Experimental group	Treatment	Number of vessels p.o.f (mean±SE)
Group A	Controls	34.61±3.57
Group B	rHuEPO	51.76±2.28*
Group C	bFGF	49.61±3.86*
Group D	aFGF	37.60±3.53
Group E	VEGF	40.48±2.34*

*$p<0.05$ Compared to controls (Group A).

Immunohistochemistry. Immunohistochemistry was performed with CD31 (NOVOCASTRA) on serial tissue sections. Tissue specimens were fixed in formalin and embedded in paraffin according to standard procedures. Four-micron sections of representative blocks from each sample were deparaffinized, dehydrated and treated with 0.3% H_2O_2 for 5 min in methanol to prevent endogenous peroxidase activity. Slides were then incubated for 75 min with the CD31 antibody at a 1:50 dilution. Control slides were incubated for the same period with normal serum (negative control). A positive control (liver tissue of mouse containing many vessels) was always run in the assay. The Envision Kit (dextran-free biotin one-step, DAKO, North America Inc, Carpinteria, CA, USA) was used according to the manufacturer's instructions. Finally, bound antibody complexes were stained for 10 min with 0.05% diaminobenzidine. Sections were then briefly counterstained with Mayer's haematoxylin, mounted and examined under a NIKON ECLIPSE 50i light microscope. High visualization fields (hot spots) were selected and the measurement of blood vessels was carried out under ×200 magnification in ten selected fields with the highest vessal density.

Statistics. The number of vessels per optical field was estimated in every specimen and is presented as mean±standard error of the mean (S.E.M.). Descriptive statistics were calculated for all five groups. The comparisons among the groups were performed using one-way ANOVA followed by Tukey's HSD posthoc Test (SPSS, v. 12).

Results

The results of our experimental study are presented in Table I. A significant increase (by 33%) in the mean number of endothelial cells in mouse myocardium tissue in group B (rHuEPO) was observed when compared to the control group A (Figure 1A, B). In addition, the mean number of endothelial cells of mouse heart of group C (bFGF) was

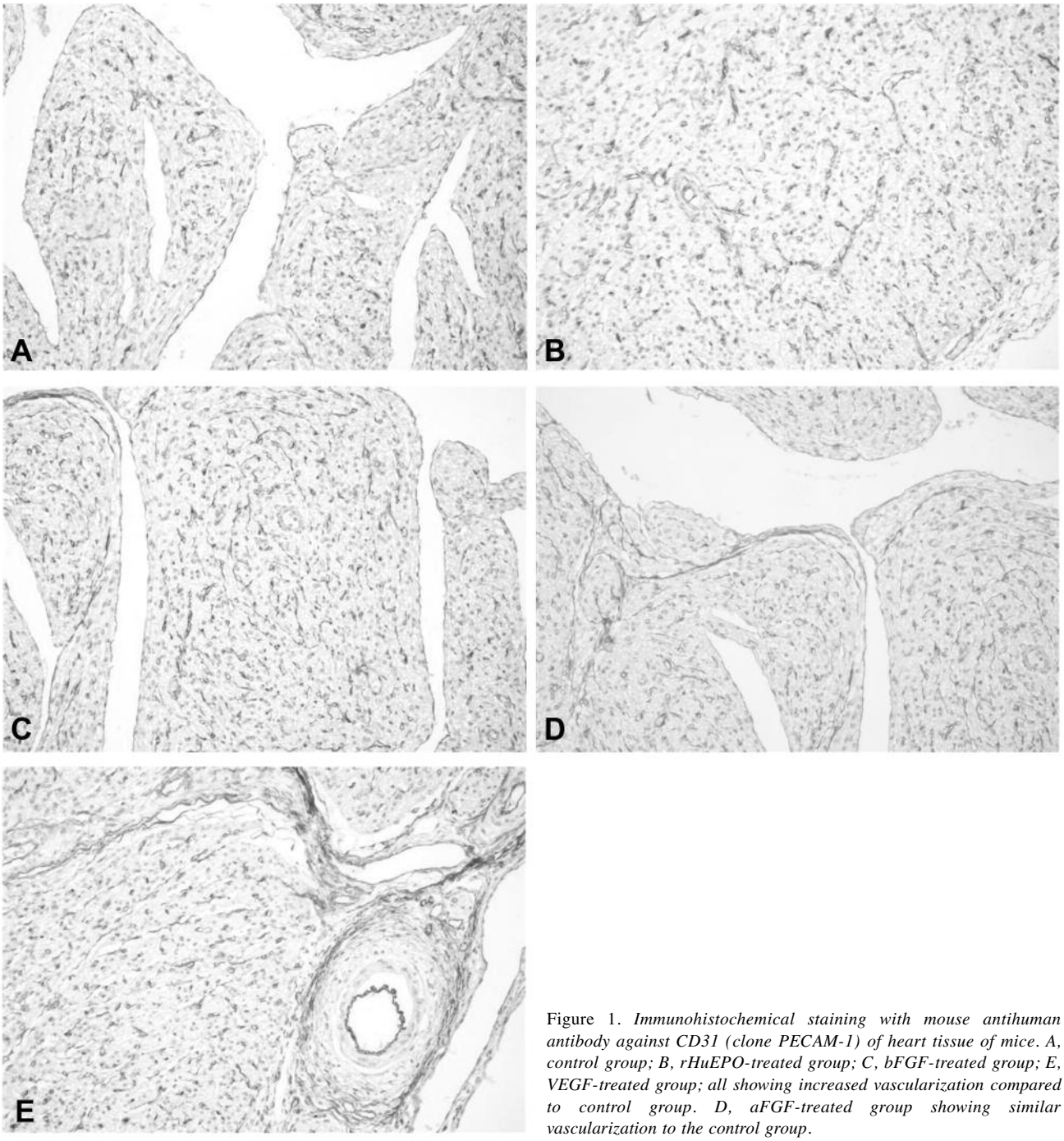

Figure 1. *Immunohistochemical staining with mouse antihuman antibody against CD31 (clone PECAM-1) of heart tissue of mice. A, control group; B, rHuEPO-treated group; C, bFGF-treated group; E, VEGF-treated group; all showing increased vascularization compared to control group. D, aFGF-treated group showing similar vascularization to the control group.*

greater (by 31%) compared to that of the controls (group A) ($p<0.05$) (Figure 1C). On the other hand, no increase was observed when comparing the number of endothelial cells between group D (aFGF) and controls (Figure 1D). In group E (VEGF-treated), the mean number of endothelial cells of mouse heart was statistically significantly increased (by 13%) compared to that of the control group A (Figure 1E).

Discussion

Since 1990, the control of erythropoiesis as the sole physiological role for EPO was challenged, as widespread expression of EPO-R and EPO was identified in endothelium (19). Further work investigated the role of erythropoietin in angiogenesis and vasculogenesis from embryonic development through adulthood (20).

The angiogenic role of EPO has been confirmed using many methods such as immunohistochemistry and immunoblotting in several tissues (21-23). However, data concerning the angiogenic effect of EPO are rather rare compared to the plethora of literature existing from other well-known angiogenic factors, such as FGFs and VEGF. To elucidate this role, we investigated the angiogenic potential of rHuEPO on endothelial cells of normal mouse myocardial tissue and compared it to that of the angiogenic cytokines aFGF, bFGF and VEGF. We demonstrated that the administration of rHuEPO increased endothelial cell proliferation in normal mouse heart.

Other investigators found that EPO stimulated capillary outgrowth in an *in vitro* assay using adult myocardial tissue thus implying a role of EPO in vasoproliferative processes (18, 24). *EPO-R* mRNA and protein can be detected in human, murine and rat hearts, with the highest level of EPO-R expression being found on endothelial cells and cardiac fibroblasts (25-27). All these findings could explain the increase of endothelial cell proliferation in normal mouse heart under rHuEPO treatment in our experiment.

The dual function of EPO, *i.e.* promotion of angiogenesis and action as a survival factor on endothelial cells, makes it interesting as a drug for the treatment of patients with ischemic disease. Nishiya *et al.* (28) found that EPO significantly enhanced angiogenesis and reduced apoptotic cell death in peri-infarcted myocardium. Moreover, if administered after myocardial infarction, EPO prevents cardiac remodelling and improves ventricular function, with enhanced angiogenesis and reduced apoptosis. In another study, a functional EPO-R was present in adult rat cardiac tissue and exogenous EPO administration improved cardiac function after ischemia/reperfusion injury (29).

We and others have already shown that growth factors such as aFGF, bFGF and VEGF mediate neovascularization in animal or human myocardial tissue. Sasame *et al.* observed significant neoangiogenesis in an experimental canine model when they administered bFGF to dogs with coronary artery occlusion (30). In an earlier study, we observed that only bFGF and VEGF mediate neovascularization in rat heart compared to controls, whereas the administration of aFGF does not increase neoangiogenesis in rat heart (13-14). In this study, we showed a similar effect of these agents in mouse heart.

Clinical trials on therapeutic angiogenesis in the myocardium have been published, since it was first demonstrated in patients with coronary heart disease to whom the growth factor FGF-1 was injected intramyocardially inducing neoangiogenesis in the myocardium (31). Ruel *et al.* also showed that the size of the ischemic defect was significantly reduced in patients receiving FGF-2 compared to placebo (32). Successful therapeutic angiogenesis has been reported with VEGF protein or genes that encode VEGF in animal models (33-37) or clinical trials (38-39).

Only Jaquet *et al.* have shown that the administration of rHuEPO had the same angiogenic potential as VEGF on adult endothelial cells derived from human myocardial tissue (18). The cardioprotective effect of EPO in animal models of myocardial infarction has been associated with increased myocardial neovascularization (Hirata *et al.* 2006, Prunier *et al.* 2007). In a recent study, Westernbrink demonstrated that EPO stimulates normal endothelial progenitor cell-mediated endothelial turnover, but cardiac microvascularization and function was improved only in the presence of ischemia (40).

In conclusion, our study demonstrated that rHuEPO induces an increase in endothelial cell proliferation in mouse myocardial tissue and also that this effect is similar to that of bFGF and VEGF. The administration of aFGF does not show angiogenic potential.

References

1 Jelkmann W: Biology of erythropoietin. Clin Invest *72(Suppl 6)*: S3-S10, 1994.

2 Anagnostou A, Liu Z, Steiner M Chin K, Lee ES, Kessimian N and Noguchi CT: Erythropoietin receptor mRNA expression in human endothelial cells. Proc Nat Acad Sci USA *91*: 3974-3978, 1994.

3 Juul SE, Anderson DK, Li Y and Christensen RD: Erythropoietin and erythropoietin receptor in the developing human central nervous system. Pediatr Res *43*: 40-49, 1998.

4 Fairchild D, Benyo D and Conrad KP: Expression of the erythropoietin receptor by trophoblast cells in the human placenta. Biol Reprod *60*: 861-870, 1999.

5 Ribatti D, Vacca A, Roccaro AM, Crivellato E and Presta M: Erythropoietin as an angiogenic factor. Eur J Clin Invest *33*: 891-896, 2003.

6 Ribatti D, Presta M, Vacca A Ria R, Giuliani R, Dell'Era P, Nico B, Roncali L and Dammacco F: Human erythropoietin induces a proangiogenic phenotype in cultured endothelia cells and stimulates neovascularization *in vivo*. Blood *93(8)*: 2627-2636, 1999.

7 Gospodarowicz D: Localization of FGF and its effect alone and with hydrocortisone on 3T3 cell growth. Nature *249*: 123-127, 1974.

8 Carmellet P: Mechanisms of angiogenesis and arteriogenesis. National Medicine *6*: 389-395, 2000.

9 Folkman J and Handernschild C: Angiogenesis *in vitro*. Nature *288*: 551-556, 1980.

10 Veikkola T and Alitalo K: VEGFs, receptors and angiogenesis. Seminars in Cancer Biology *9*: 211-220, 1999.

11 Muller YA, Li B, Christinger HW, Wells JA, Cunningham BC and deVos AM: Vascular endothelial growth factor: crystal structure and functional mapping of the kinase domain receptor binding site. Proc Natl Acad Sci USA *94*: 7192-7197, 1997.

12 Shweiki D, Itin A, Soffer D and Keshet E: VEGF induced by hypoxia may mediate hypoxia initiated angiogenesis. Nature *359*: 843-845, 1992.

13 Efthimiadou A, Asimakopoulos B, Nikolettos N, Giatromanolaki A, Sivridis E, Lialiaris TS, Papachristou DN and Kontoleon E: The angiogenic effect of intramuscular administration of VEGF on muscle. The influence of exercise on angiogenesis. In Vivo *18(6)*: 825-829, 2004.

14 Efthimiadou A, Asimakopoulos B, Nikolettos N, Giatromanolaki A, Sivridis E, Lialiaris TS, Papachristou DN and Kontoleon E: The angiogenic effect of intramuscular administration of b- FGF and aFGF on cardiac muscle. The influence of exercise on muscle angiogenesis. J Sports Sci *24(8)*: 849-54, 2006.
15 Unger EF, Banai S and Shou M: bFGF enhances myocardial collateral flow in a canine model. Am J Physiol M 226 (Heart Circulation Physiology) *35*: H1588-1595, 1994.
16 Safi JJr, DiPaula AFJr, Riccioni T, Kajstura J, Ambrosio G, Becker LC, Anversa P and Capogrossi MC: Adenovirus-mediated *acidic FGF* gene transfer induces angiogenesis in the nonischemic rabbit heart. Microvasc Res *58*: 238-249, 1999.
17 Ibukiyama C: Angiogenic therapy using FGF and VEGF for ischemic vascular lesions. Jpn Heart J *137*: 285-300, 1996.
18 Jaquet K, Krause K, Tawakol-Khodai M Geidel S, Kuck KH: Erythropoietin and VEGF exhibit equal angiogenic potential. Microvasc Res *64*: 326-333, 2002.
19 Anagnostou A, Lee ES, Kessimian N, Levinson R and Steiner M: Erythropoietin has a mitogenic and positive chemotactic effect on endothelial cells. Proc Natl Acad Sci USA *87*: 5978-5982, 1990.
20 Muller-Ehmsen J, Schmidt A, Krausgrill B, Schwinger RH and Bloch W: Role of erythropoietin for angiogenesis and vasculogenesis: from embryonic development through adulthood. Am J Physiol Heart Circ Physiol *290(1)*: H331-340, 2006.
21 Carlini RG, Reyes A and Rothstein M: Recombinant human erythropoietin stimulates angiogenesis *in vitro*. Kidney Int *47*: 740-745, 1995.
22 Galeano M, Altavilla D, Bitto A, Minutoli L, Calò M, Lo Cascio P, Polito F, Giugliano G, Squadrito G, Mioni C, Giuliani D, Venuti FS and Squadrito F: Recombinant human erythropoietin improves angiogenesis and wound healing in experimental burn wounds. Crit Care Med *34(4)*: 1139-1146, 2006.
23 Westenfelder C, Biddle DL and Baranowski RL: Human, rat and mouse kidney cells express functional erythropoietin receptors. Kidney Int *55*: 808-820, 1999.
24 Kertesz N, Wu J, Chen TH, Sucov HM and Wu H: The role of erythropoietin in regulating angiogenesis. Dev Biol *276(1)*: 101-110, 2004.
25 Depping R, Kawakami K, Ocker H, Wagner JM, Heringlake M, Noetzold A, Sievers HH and Wagner KF: Expression of the erythropoietin receptor in human heart. J Thorac Cardiovasc Surg *130*: 877-878, 2005.
26 Wright GL, Hanlon P, Amin K Steenbergen C, Murphy E and Arcasoy MO: Erythropoietin receptor expression in adult rat cardiomyocytes in associated with an acute cardioprotective effect for recombinant during ischemia-reperfusion injury. FASEB J *18*: 1031-1033, 2004.
27 Sharples JS, Thiemermann C and Yaqoob M: Novel applications of recombinant erythropoietin. Curr Opin Pharmacol *6*: 184-189, 2006.
28 Nishiya D, Omura T, Shimada K, Matsumoto R, Kusuyama T, Enomoto S, Iwao H, Takeuchi K, Yoshikawa J and Yoshiyama M: Effect of erythropoietin on cardiac remodelling after myocardial infarction. J Pharmacol Sci *101*: 31-39, 2006.
29 van der Meer P, Lipsic E, Henning RH, de Boer RA, Suurmeijer AJ, van Veldhuisen DJ and van Gilst WH: Erythropoietin improves left ventricular function and coronary flow in an experimental model of ischemia-reperfusion injury. Eur J Heart Failure *6*: 853-859, 2004.
30 Sasame A, Nakajima H, Tamura K, Miyagi M, Rakue H, Usui M, Katoh T, Naitoh Y and Ibukiyama C: A study to determine if bFGF reduces myocardial infarct size in acute coronary arterial occlusion Jpn Heart *40*: 165-178, 1999.
31 Schumacher B, Pecher P, von Specht BU and Stegmann T: Induction of neoangiogenesis in ischemic myocardium by human growth factors: first clinical results of a new treatment of coronary heart disease Circulation *97*: 645-650, 1998.
32 Ruel M, Laham RJ, Parker JA, Post MJ, Ware JA, Simons M and Sellke FW: Long-term effects of surgical angiogenic therapy with fibroblast growth factor 2 protein. J Thorac Cardiovasc Surg *124*: 28-34, 2002.
33 Banai S, Jaklitsch MT, Shou M, Lazarous DF, Scheinowitz M, Biro S, Epstein SE and Unger EF: Angiogenic-induced enhancement of collateral blood flow to ischemic myocardium by vascular endothelial growth factor in dogs. Circulation *89*: 2183-2189, 1994.
34 Harada K, Friedman M, Lopez JJ, Wang SY, Li J, Prasad PV, Pearlman JD, Edelman ER, Sellke FW and Simons M: Vascular endothelial growth factor administration in chronic myocardial ischemia Am J Physiol *270(5 pt 2)*: H1791-H1802, 1996.
35 Lopez JJ, Laham RJ, Stamler A, Pearlman JD, Bunting S, Kaplan A, Carrozza JP, Sellke FW and Simons M: VEGF administration in chronic myocardial ischemia in pigs. Cardiovasc Res *40*: 272-281, 1998.
36 Hariawala MD, Horowitz JR, Esakof D, Sheriff DD, Walter DH, Keyt B, Isner JM and Symes JF: VEGF improves myocardial blood flow but produces EDRF-mediated hypotension in porcine hearts. J Surg Res *63*: 77-82, 1996.
37 Giordano FJ, Ross J and Peterson KL: Intravenous or intracoronary VEGF ameliorates chronic myocardial ischemia. Circulation *98(suppl I)*: I-455, 1998.
38 Henry TD, Annex BH, Mckendall GR Azrin MA, Lopez JJ, Giordano FJ, Shah PK, Willerson JT, Benza RL, Berman DS, Gibson CM, Bajamonde A, Rundle AC, Fine J, McCluskey ER; VIVA Investigators: The VIVA trial: VEGF in ischemia for vascular angiogenesis. Circulation *107(10)*: 1359-1365, 2003.
39 Annex BH and Simons M: Growth factor-induced therapeutic angiogenesis in the heart: protein therapy. Cardiovasc Res *65(3)*: 649-655, 2005.
40 Westenbrink BD, Oeseburg H, Kleijn L, van der Harst P, Belonje AM, Voors AA, Schoemaker RG, de Boer RA, van Veldhuisen DJ and van Gilst WH: Erythropoietin stimulates normal endothelial progenitor cell-mediated endothelial turnover, but attributes to neovascularization only in the presence of local i/schemia. Cardiovasc Drugs Ther, 2008, in press.

Received March 3, 2008
Revised May 29, 2008
Accepted June 5, 2008